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Original Article

On the effect of the injection of potassium phosphate *in vivo* inducing the precipitation of serum calcium with inorganic phosphate

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Abstract: High concentrations of inorganic phosphate (Pi) resulted from the hydrolysis of ATP is strongly associated to the weakness of the contractile mechanism of muscles due to its attractiveness to calcium. The majority of the experiments to study such effect are conducted *in vitro*. This work investigates the effects of different concentrations of Pi, induced by the injection of potassium phosphate in live animals, in the precipitation with serum calcium and the generation of calcium phosphate composites. The experiments were also designed to find out the ideal amount of potassium phosphate to induce an effective reaction. Potassium phosphate was injected in Wistar rats, randomly separated and distributed into seven groups. Group I was injected with 0.5 ml of saline solution (control) and groups II through VII were injected with 0.5, 1.5, 2.5, 5.0, 7.5 and 10.0 mg/kg of potassium phosphate, respectively. Blood collected from the inferior vena cava was submitted to biochemical analyses to measure the concentrations of calcium, Pi, urea and creatinine. The results showed that Pi, induced by the injection of potassium phosphate in live animals, causes precipitation with serum calcium, with statistically significant differences between the control and the treatment groups for doses up to 5.0 mg/kg. No statistically significant differences were found between the different doses and the concentration of urea and creatinine in the plasma. We conclude that potassium phosphate can be used to induce serum calcium precipitation *in-vivo*, with minor effects on other physiological variables, and the ideal dose to do so is 5.0 mg/kg.

Keywords: In vivo, potassium phosphate, inorganic phosphate, serum calcium, muscle fatigue

Introduction

The effect of extracellular calcium and inorganic phosphate (Pi) on various aspects of the cellular mechanism has been the object of study of many research groups over the past decades [1-7]. Special attention has been devoted to the role of those substances in the underlying process of skeletal muscle contraction that begins with the release of a series of action potentials, promoting cell membrane depolarization and the release of calcium ions from the sarcoplasmic reticulum terminal [8-10]. Since the hydrolysis of adenosine triphosphate (ATP) is also required to provide energy and maintain the contraction, it is expected that the amount of ATP and its hydrolysis must be directly proportional to the demands of each contraction. Depending on the intensity of the contraction, the hydrolysis of ATP can increase the concentration of cytoplasmic Pi up to 30 mM [11-13]. Although other biochemicals, including phosphocreatine (PCr), creatine, glucose 6-P and lactate also play an important role in the regulation of muscle contraction and power generation, high concentration of cytoplasmic Pi has been reported as one of the most important element contributing to fatigue and reduction of power [5, 11, 13, 14]. This can be explained by the attractiveness of Pi to calcium ions (Ca^{2+}), released by the sarcoplasmic reticulum (SR), leading to the formation of an insoluble substance called calcium phosphate (CaPi) and reducing the quantities of Pi and Ca^{2+} required to sustain the contraction [3].

Fryer *et al* were the first to suggest that Pi could enter the sarcoplasmic reticulum and precipi-

tate Ca^{2+}, forming CaPi and rapidly reducing the amount of calcium ions released, contributing to muscle fatigue [1]. Other works can also be found in the literature describing the effects of Pi reacting with Ca^{2+} released by the sarcoplasmic reticulum (SR) [4, 15-18]. However, since the majority of those experiments were carried out *in vitro*, it is possible that we are missing important metabolic reactions and substances, that could not be found or replicated *in vitro* but would play a significant role in the process of muscle contraction. Therefore, one can infer that the results of those surveys, while worthy of attention, are somehow limited to specific areas of interest. In fact, Fryer *et al* conclude that the omission of some possible *in vivo* reactions did not allow them to clearly establish if the decreased muscle strength was only due to precipitation of CaPi within the sarcoplasmic reticulum, or due to the loss of Ca^{2+} by the SR [1]. For example, cytoplasmic Pi can increase the probability of opening SR's ryanodine receptors (RyRs) [12, 19, 20]. RyRs are vital for muscle contraction since they are responsible for releasing Ca^{2+} into the cytosol [7, 21]. Hence, if cytoplasmic Pi can change the probability of opening RyRs channels, it can interfere with the whole process of muscle contraction. Also, cytoplasmic Pi may enter the SR and bind to Ca^{2+}, forming CaPi, further reducing the amount of releasable Ca^{2+} [6]. Nevertheless, it is possible that muscle strength is not affected by a significant reduction in Ca^{2+} available in the SR, since its endogenous contents of calcium (~1.1 mmol/l) may be much greater than necessary to fully activate the contractile mechanism [2, 9]. However, this is yet to be demonstrated.

In this article, we propose a method that can potentially be used to verify some of those important reactions *in vivo*. We investigate whether inorganic phosphate, induced by the injection of potassium phosphate (K_3PO_4) in live animals, causes serum calcium precipitation, generating the insoluble calcium phosphate and, if so, what is the ideal amount to do it effectively. If the method proves to be feasible, without prejudice to the animal's health, it can contribute to the opening of new frontiers to the study of muscle fatigue in animals or humans.

Materials and methods

Animals

Male rats of the Wistar strain, with an average age of 90 to 120 days, weighing between 50 and 150 gr, maintained on food and water *ad libitum*, housed in cages of white polyethylene (42x24x17 cm), with a maximum of five animals per cage, with daily hygiene, under controlled lighting conditions and light/dark cycle of 12/12 hours. The animals were acclimatized in the laboratory for at least 1 h. All experiments were conducted between 11:00 h and 17:00 h in order to minimize possible circadian variations [22].

Very old and very young animals or those presenting any disease that could affect the energetic or contractile capacity were excluded.

Groups

The animals were randomly separated and distributed into seven groups of seven animals each: Group I (control – sterile saline solution), Group II (K_3PO_4-0.5 mg/kg), Group III (K_3PO_4-1.5 mg/kg), Group IV (K_3PO_4-2.5 mg/kg), Group V (K_3PO_4-5.0 mg/kg), Group VI (K_3PO_4-7.5 mg/kg) and Group VII (K_3PO_4-10.0 mg/kg). As shown, the difference between the groups is the amount of potassium phosphate administered via rapid injection (for Groups II, III, IV, V, VI and VII), except for Group I, used as control and injected with sterile saline solution. In order to rule out the effect of animal handling or features of the vehicle used for drug administration, and to establish a base line for the analyses, the control group was injected with 0.5 ml of sterile saline solution (NaCl 0.9%).

It is important to emphasize that potassium phosphate is a substance widely used to control hypophosphatemia [23-27]. The amounts used in the experiments were based on the specifications given by Gaasbeek and Meinders [28], that used infusions of potassium phosphate at doses of 2.5 mg/kg.

After inoculation, the animals were kept in their cages for 10 minutes prior to the surgical procedure for blood sampling, with no restriction to movement. A previous pilot experiment performed by the authors showed that the concentrations of serum calcium, Pi, urea and creatinine were stable 10 minutes after the injection of different doses of K_3PO_4.

Surgical procedure

The animals were weighed and anesthetized with an intraperitoneal injection [29] of Sodium Pentothal 70 mg/kg (Anental®). They were considered anesthetized when there was no

response to mechanical stimuli and no movement was observed after a painful stimulus - gripping the tail. The animals were placed on a metal tray in the supine position, with pelvic and thoracic limbs restrained by adhesive tape. A median longitudinal incision, of approximately 8 cm, was made in the abdominal region with fine scissors, with exposure and lateral spacing of the bowel to the right, allowing the visualization of the inferior vena cava and withdrawal of venous blood, which was collected and conditioned in tubes without coagulants and centrifuged to obtain the serum (Bio Eng® Serum Centrifuge, Model BE-5000 no 1616). The serum was separated from the cellular portion of the blood no later than twenty minutes after collection, avoiding ion exchanges between the two due to the increased permeability of erythrocytes to calcium [30] and possible release of hematic phosphate [31], which could affect the results of the biochemical analysis. Hemolyzed blood samples were excluded from the biochemical analysis.

Biochemical analysis

The concentrations of calcium, inorganic phosphate, urea and creatinine in the blood were measured threefold, and the biochemical values were used to represent the serum amounts of the substances. Blood samples were collected with Semi-automatic pipette (Digipet® 1000 µl) and analyzed by the following methods:

Calcium: Cálcio-K007 (Bioclin®) - End point colorimetric test.

This is a colorimetric test for in vitro diagnostic, based on the measured of the intensity of color produced by the compound formed by o-cresolphthalein complexone and calcium in alkaline pH. The concentration of calcium can be calculated by measuring the absorbance of the sample [32]. More details about this technique can be found in [30].

Inorganic phosphate: Fósforo-K020 (Bioclin®) - End point colorimetric test.

This test is based on the reaction of Pi with ammonium molybdate, forming ammonium phosphomolybdate, which is subsequently reduced to molybdenum-blue - a blue substance, which the intensity of color is proportional to the concentration of Pi [32]. By measuring the absorbance of the samples it is possible to infer the concentration of Pi. A detailed description of the method is given by [31].

Creatinine: Creatinina-K0106 (Bioclin®) - End point colorimetric test (Jaffe modified).

It is a colorimetric test for in vitro diagnostic, based on the reaction of creatinine with picric acid, forming a reddish-yellow complex. In this pH occurs the maximum formation of the picric-creatinine color complex. By adding the acid reagent, pH is reduced and the color due to creatinine is undone, remaining the chromogens color [33]. The concentration of creatinine can be obtained by the difference between the absorbances at different pHs. A full description of the method can be found in [34].

Urea: Uréia Enzimática-K047 (Bioclin®) - End point enzymatic colorimetric test (Berthelot modified).

This test is based on the principle that urea can be hydrolyzed to ammonium ions and CO_2 by urease ($urea + 3H_20 \rightarrow^{urease} 2NH_4^+ + CO_2 + 2OH^-$). At alkaline pH and in the presence of salicylate and sodium hypochlorite, ammonia reacts and results in a compound of green color whose intensity is proportional to the concentration of urea in the sample [33]. Therefore, by measuring the absorbance of the samples it is possible to infer the concentration of urea. A full description of method the can be found in [35].

The study was approved by the Ethics Committee for Animal Experimentation of the Federal University of Minas Gerais, Brazil, according to Protocol #020.

Results

Dose-response curves were calculated for different doses of potassium phosphate versus the concentration of calcium, inorganic phosphate, urea and creatinine found in the plasma. The analyses of the dose-response curves were based on a graphical observation of the physiological responses and trend analysis. The tests of hypotheses were based on the average concentration of the substances in the blood, followed by a further analysis of trends. **Table 1** shows the Linear (1-2) and Nonlinear (3-5) models tested for trend analyses.

Table 1. Linear and nonlinear regression models tested when searching for best-fit method for the dose-response curves

1	Linear	$y_i=y_0+ax$
2	Quadratic	$y_i=y_0+ax+bx^2$
3	Negative exponential	$y_i=ae^{-bx}$
4	Negative exp. of three parameters	$y_i=y_0+ae^{-bx}$
5	Sigmoidal of four parameters	$y_i = y_0 + \frac{a}{1 + e^{-\left\lvert\frac{x-x_0}{b}\right\rvert}}$

A model should be selected by taking into consideration it's fitting to the data and also to the objectives of the study. In other words, prior to any consideration, a model would be selected according to: a) the biological logics - the objective, the nature, the scope and extension of the study, and the expected results; b) the significance of the adjustment of the model to the data; and c) the coefficient of determination (R^2). If more than one model would apply - expressing a functional relationship between the studied variables and allowing an estimate of the parameters, capable of defining such relationship - the coefficient of determination and the biological logics expressed by them were was also considered in order to select the most suitable model. In so doing, the chosen model would not only be a good statistical model, but also would generate the best representation of the biological aspects involved [36, 37].

Also, prior to the parametric analysis, each variable was tested for normality, independence of errors and homoscedasticity. The following procedure was used: (1) Kolmogorov-Smirnov normality test [38]; (2) Hartley's Fmax homoscedasticity test [39]; (3) Regression analyses with all models listed in **Table 1**; (4) Visual analysis of trends - adjustment of the model to the data, by means of a graph showing the mean values and errors.

After those tests and the preliminary graphic evaluation were concluded, it was found that all data could be represented by a normal distribution function (Calcium: K-S=0.12569, p< 0.20; Inorganic phosphate: K-S=0.167401, p< 0.15; Urea: K-S=0.11963, p< 0.20; Creatinine: K-S=0.16510, p< 0.15) and showed homogeneity of variance (Calcium: Fmax=2.9220, p< 0.1382; Inorganic phosphate: Fmax=5.87, p< 0.10; Urea: Fmax=2.2987, p< 0.1803; Creatinine: Fmax=4.25, p< 0.10).

Serum calcium concentration

Table 2 shows the estimated values for each parameter of the models, adjusted to the dose-response curve of serum calcium, according to the criteria described earlier. Note that all models show a good adjustment to the data (p< 0.05). However, when we compare the variation of the data obtained from the models and the total span of the real data, expressed by R^2, it was found that three of models showed a better adjustment (quadratic, sigmoidal and negative exponential). Nevertheless, the biological logic (the main criterion of choice) is based on the assumption that there may be a stabilization of calcium precipitation with inorganic phosphate. When testing such hypothesis, it was found that the sigmoidal regression is the best model to support it.

The graph in **Figure 1** represents the average of serum calcium for different doses of potassium phosphate. A reduction in the concentration of serum calcium can be observed as the doses of potassium phosphate increases up to 5.0 mg/kg, with high statistically significant differences. Above that value, there is a tendency towards stabilization of the response, with no statistically significant differences, as shown in **Table 3**. The continuous line shows the tendency (trend) estimated by the sigmoidal model.

Table 3 shows the comparison between the mean values of serum calcium concentration for different doses of potassium phosphate. The values marked with '*' are those that, according to the test, do not differ significantly, with a confidence level of 95%. Note that the control group (0.0 mg/kg of potassium phosphate) is significantly different from the others. The same can be seen for doses of 0.5, 1.5 and 2.5 mg/kg. Doses of 5.0, 7.5 and 10.0 mg/kg are statistically similar to each other, meaning that the concentration of serum calcium must have reached a stable level at 5.0 mg/kg of injected potassium phosphate. This effect can also be observed in **Figure 1**.

A similar strategy was used to evaluate the correlation between the doses of potassium phosphate and the concentration of inorganic phos-

Table 2. Estimated values for the parameters of the mathematical models, calculated for the serum calcium x potassium phosphate dose-response

Regression model	Y_0	a	b	x_0	R^2	$R^2_{aj.}$	p
Linear	7.9430	-0.3180			0.7579	0.7095	0.0108
Quadratic	8.6239	-0.9604	0.0662		0.9841	0.9761	0.0003
Negative exponential		8.1247	0.0539		0.8090	0.7708	0.0058
Neg. exp. of three parameters	5.2387	3.5702	0.4046		0.9629	0.9444	0.0014
Sigmoidal of four parameters	5.3532	4.0662	-1.1411	1.5231	0.9744	0.9489	0.0069

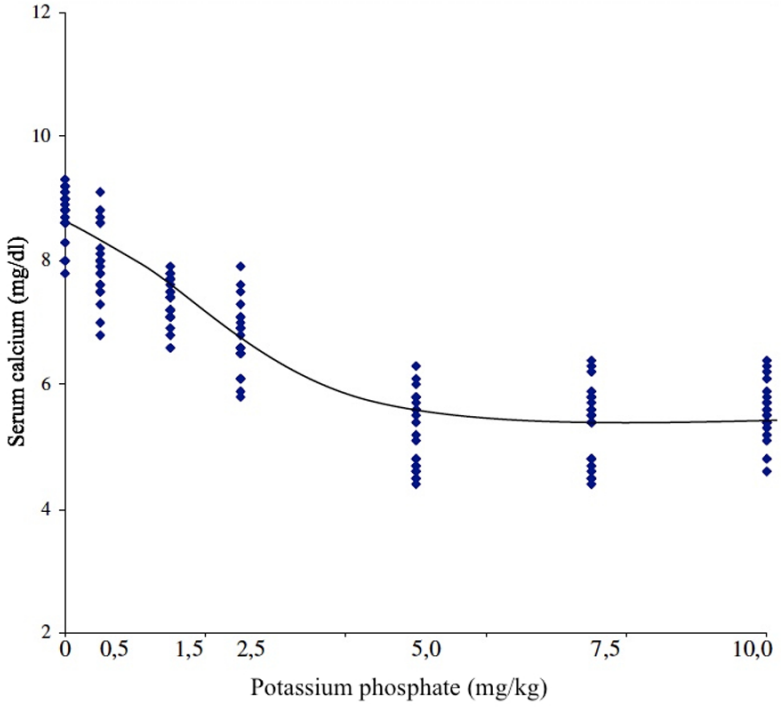

Figure 1. Relationship between doses of potassium phosphate (mg/kg) and serum calcium (mg/dl) and the trend line (four-parameter sigmoidal model).

Table 3. Results of the Tukey test comparing the mean serum calcium (mg/dl) with different doses of potassium phosphate (mg/kg)

Potassium phosphate (mg/kg)	Serum calcium (mg/dl)
0.0	8.73
0.5	7.99
1.5	7.36
2.5	6.77
5.0	5.22*
7.5	5.37*
10.0	5.57*

Values marked with '*' do not differ significantly, with confidence level of 95%.

phate, urea and creatinine in the plasma. The final results as shown next.

Serum inorganic phosphate concentration

Figure 2 shows the average concentration of inorganic phosphate (Pi) found in the blood for different doses of potassium phosphate. An increase in the concentration of Pi can be observed as the doses of potassium phosphate increases up to 2.5 mg/kg, with high statistically significant differences. Above that value, there is a tendency towards stabilization of the response, with no statistically significant differences, as shown in **Table 4**. The continuous line shows the tendency (trend) estimated by the sigmoidal model.

Serum urea concentration

Although the data from urea analyses show a normal distribution and homogeneity of variance, none of the tested regression models showed statistical significance to represent the changes of urea as a function of the dosage of inorganic phosphate. Also, no statistically significant differences were found between different doses of potassium phosphate, with a confidence level of 95%. **Figure 3** shows the average of serum urea for different doses of potassium phosphate.

Serum creatinine concentration

In the same way as for urea, although the creatinine analyses showed a normal distribution and homogeneity of variance, none of the tested regression models showed statistical signifi-

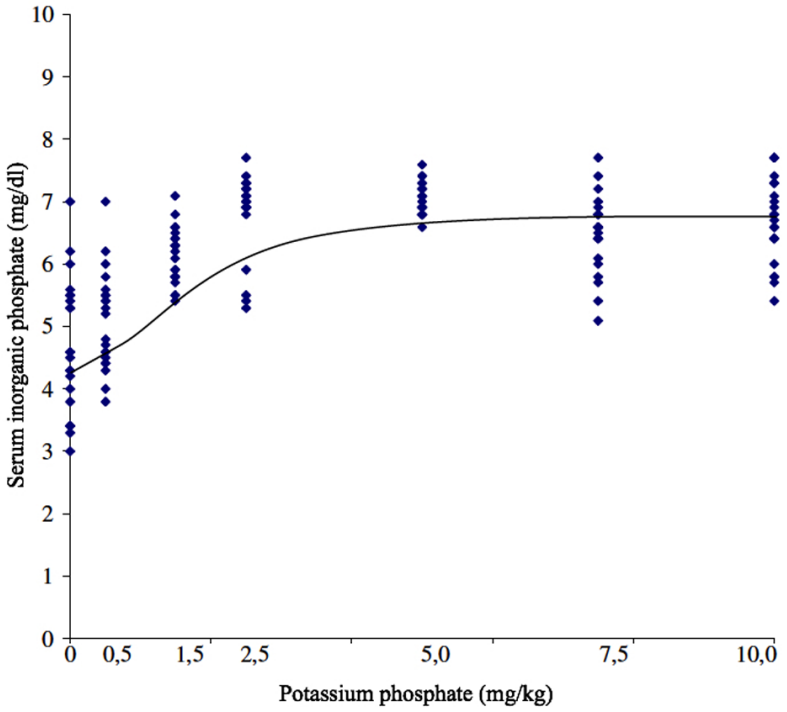

Figure 2. Relationship between doses of potassium phosphate (mg/kg) and serum Pi (mg/dl) and the trend line (four-parameter sigmoidal model).

Table 4. Results of the Tukey test comparing the mean serum Pi (mg/dl) for different doses of potassium phosphate (mg/kg)

Potassium phosphate (mg/ kg)	Serum Pi (mg/dl)
0.0	4.72
0.5	5.10
1.5	6.10
2.5	6.82*
5.0	7.06*
7.5	6.49*
10.0	6.66*

Values marked with '*' do not differ significantly, with confidence level of 95%.

cance to represent the changes in serum concentration as a function of the dosage of inorganic phosphate. No statistically significant differences were found between different doses of potassium phosphate, with a confidence level of 95%. **Figure 4** shows the average of serum creatinine for different doses of potassium phosphate.

Discussion

In this paper we have described a method for *in vivo* precipitation of calcium phosphate by means of potassium phosphate inoculation. Unlike the *in vitro* approach, predominant for this kind of experiment, the method allowed us to avoid the omission of substances that could play important roles during *in vivo* experiments, as described earlier. Also, the injection of potassium phosphate can be done with minor effects on other physiological variables that could affect the desired results, allowing a new spectrum of researches to better understand the processes of muscle contraction and muscle fatigue.

The experimental setup consisted on the intraperitoneal injection of different doses of potassium phosphate and biochemical analyses of blood collected from the inferior vena cava of the animals. Although we did not performed *in vitro* experiments, it is assumed that the precipitation of calcium phosphate occurred in a similar manner to the reactions reported by other *in vitro* experiments described in the literature [12, 16, 17].

The results show that injection of potassium phosphate raises the concentration of inorganic phosphate in the blood for doses up to 2.5 mg/kg - higher doses have no significant impact in the concentration of serum Pi. The amount of serum calcium was substantially reduced after the injection of potassium phosphate. Such reduction also varied according to the dosage of potassium phosphate, with no statistically significant changes for doses above 5.0 mg/kg. We assume that the dose-response has reached a certain metabolic equilibrium at 5.0 mg/kg. Confirming this assertion, the tests of trend analysis showed that the concentrations of serum calcium in response to inoculation of doses of 5.0, 7.5 and 10.0 mg/kg of potassium phosphate were basically the same (**Figure 1**, **Table 3**). When comparing the responses of calcium concentration between the control group (0.0 mg/kg of potassium phosphate) and the

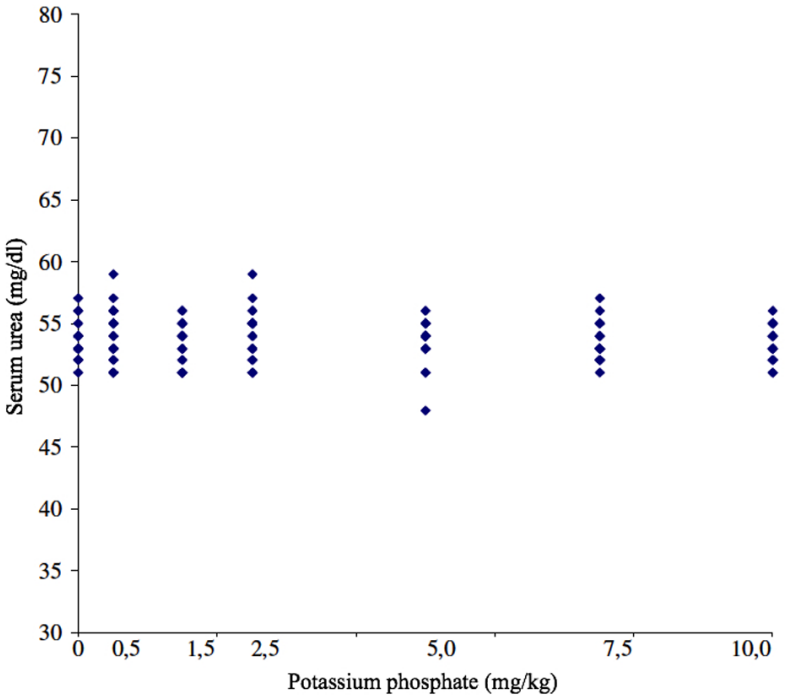

Figure 3. Relationship between doses of potassium phosphate (mg/kg) and serum urea (mg/dl).

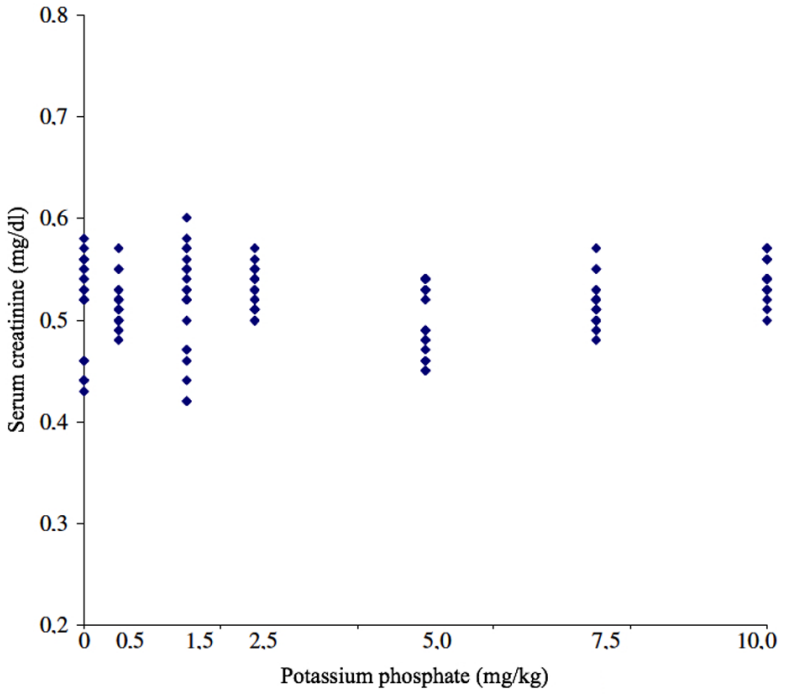

Figure 4. Relationship between doses of potassium phosphate (mg/kg) and serum creatinine (mg/dl).

Another important result gathered with this study was that the injection of potassium phosphate, at doses up to 10 mg/kg, did not have any significant impact in important metabolic functions during the experiments. It is known that changes in urea and creatinine may indicate abnormal functioning of the urinary system and protein metabolism [40]. Although there is some debate on the efficiency of those markers as indicators of premature renal damage, it is a consensus that high concentration of those substances in the plasma may indicate a variety of renal dysfunctions and damage [40, 41]. One might rightfully wonder whether high levels of urea could also be the result of the degradation of exogenous proteins from a feeding process. However, that was not the case of this study, since the animals were kept fasting prior to the experiments. High levels of creatinine could also be the result of intense physical activity or muscular lesions [42, 43], but again, to avoid such effect, the animals were not stressed prior to the experiments. Therefore, the absence of changes in the concentration of serum urea and creatinine (**Figures 3** and **4**) indicates that the injection of potassium phosphate did not have major effects on physiological variables that could be traced by urea or creatinine, for the duration of the experiments.

experimental Group V (5.0 mg/kg - promoter of the maximum effect, as shown in **Table 3**), a highly significant difference was observed.

By proving a method to induce the precipitation of calcium phosphate in live animals and defining and ideal dose to do so (5.0 mg/kg in rats),

we hope to provide a novel research tool for studying the role of inorganic phosphate and calcium concentration in various physiological processes, in particular, those related to skeletal muscle fatigue.

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Disclosure of conflict of interest

None.

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